Development 126, 3313-3322 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV5319

Differential interactions between Brother proteins and Runt domain proteins in the *Drosophila* embryo and eye

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Accepted 20 May; published on WWW 5 July 1999

SUMMARY

Brother and Big brother were isolated as Runt-interacting proteins and are homologous to CBFβ, which interacts with the mammalian CBFα Runt-domain proteins. In vitro experiments indicate that Brother family proteins regulate the DNA binding activity of Runt-domain proteins without contacting DNA. In both mouse and human there is genetic evidence that the CBFα and CBFβ proteins function together in hematopoiesis and leukemogenesis. Here we demonstrate functional interactions between Brother proteins and Runt domain proteins in *Drosophila*. First, we show that a specific point mutation in Runt that disrupts interaction with Brother proteins but does not affect DNA binding activity is dysfunctional in several in vivo assays. Interestingly, this mutant protein acts dominantly to interfere with the Runt-dependent activation of *Sxl-lethal* transcription. To investigate further the requirements for Brother proteins in *Drosophila* development, we examine the effects of expression of a Brother fusion protein homologous to the dominant negative CBFβ::SMMHC fusion protein that is associated with leukemia in humans. This Bro::SMMHC fusion protein interferes with the activity of Runt and a second Runt domain protein, Lozenge. Moreover, we find that the effects of *lozenge* mutations on eye development are suppressed by expression of wild-type Brother proteins, suggesting that Brother/Big brother dosage is limiting in this developmental context. Results obtained when Runt is expressed in developing eye discs further support this hypothesis. Our results firmly establish the importance of the Brother and Big brother proteins for the biological activities of Runt and Lozenge, and further suggest that Brother protein function is not restricted to enhancing DNA-binding.

Key words: *runt*, *lozenge*, *AML1*, CBFβ::MYH11, Inv(16), *Sex-lethal*, *engrailed*

INTRODUCTION

Runt domain proteins are a recently described family of transcriptional regulators with pivotal roles in several developmental pathways in *Drosophila*, mammals and *Xenopus* (for reviews see Speck and Stacy, 1996; Wang et al., 1996a; Daga et al., 1996; Ducy et al., 1997; Kramer et al., 1998; Tracey et al., 1998). The family members are defined by the Runt domain, a 128 amino acid region that mediates sequence-specific DNA binding (Ogawa et al., 1993a; Bae et al., 1994; Kagoshima et al., 1996). Significant insights on the functions of Runt domain proteins came from studies on a mammalian transcription factor referred to as polyomavirus enhancer-binding protein 2 (PEBP2) or as core binding factor (CBF) (Ogawa et al., 1993a; Wang and Speck, 1992). This transcription factor, referred to here as CBF, has important roles in leukemogenesis, hematopoiesis and osteogenesis (Tanaka et al., 1995; Miyoshi et al., 1995; Okuda et al., 1996; Wang et al., 1996a,b; Komori et al., 1997; Otto et al., 1997). It regulates the expression of genes specific to T-, B-, myeloid or osteoblast cell lineages through DNA binding (for reviews, see Speck and Stacy, 1996; Geoffroy et al., 1995; Ducy et al., 1997). Cloning of cDNAs for CBF revealed that it is a heteromeric complex comprised of two unrelated proteins (Ogawa et al., 1993b; Wang et al., 1993). The DNA-binding α subunit proteins are Runt-domain proteins while the β subunit is a novel protein. CBFβ does not bind to DNA, but instead interacts with the Runt domain to enhance DNA-binding affinity by an unknown mechanism (Ogawa et al., 1993b; Crute et al., 1996).

Several lines of evidence indicate that CBFα and CBFβ proteins are likely to function together to regulate the growth and/or differentiation of hematopoietic progenitors in mammals. The human Runt domain gene *AML1* is expressed in a number of lymphoid cell lines and is a frequent target for chromosomal translocations associated with acute myeloid leukemias (Miyoshi et al., 1993, 1995; de Greef et al., 1995). Interestingly, chromosomal rearrangement of the human *Cbfb* gene is also associated with a subtype of acute myeloid leukemia (Liu et al., 1993; Claxton et al., 1994; Marlton et al., 1995). In this case the rearrangement is an inversion of chromosome 16, which results in a fusion protein containing

most of CBFβ at the N terminus and the coiled-coil tail region of the smooth muscle myosin heavy chain *MYH11* gene at the C terminus. Cell culture experiments suggest that CBFβ::MYH11 disrupts CBF function by sequestering the normally nuclear CBFα subunit in the cytoplasm (Kanno et al., 1998; Adya et al., 1998).

Experiments in the mouse system provide further evidence that CBFα and CBFβ function together in hematopoiesis. First, the phenotypes associated with knock-out mutations of *Cbfa* and *Cbfb* are nearly identical. Homozygous disruption of either gene results in hemorrhaging in the central nervous system, and blocks fetal liver hematopoiesis (Wang et al., 1996a,b; Okuda et al., 1996). Similar phenotypes are also obtained in mice with one copy of the knocked-in *Cbfb::MYH11* gene (Castilla et al., 1996). These several observations strongly suggest that the CBFα and CBFβ proteins function together in vivo, presumably as a DNA-binding heterodimeric regulator.

There are two well-characterized Runt-domain proteins in *Drosophila*, Runt and Lozenge (Lz). The *runt* gene participates in three developmental pathways: sex determination, segmentation and neurogenesis (Gergen and Wieschaus, 1986; Torres and Sanchez, 1992; Duffy et al., 1991; Duffy and Gergen, 1991; Dormand and Brand, 1998). In the sex determination pathway Runt directly activates *Sex-lethal* (*Sxl*) gene expression (Kramer et al., 1998), while in the segmentation pathway Runt acts as both an activator and a repressor of segmentation gene expression (Tsai and Gergen, 1994; Aronson et al., 1997). Lz plays an important role in patterning the developing eyes (Daga et al., 1996; Flores et al., 1998). It is absolutely required in R7 and cone cell precursors to negatively regulate *seven-up* expression and functions in R1/R6 photoreceptors to positively regulate *Bar* expression. Although some of the target genes regulated by Runt and Lz have been identified, the mechanism by which these two proteins regulate the expression of their target genes is not fully understood.

Two *Drosophila* CBFβ homologues, referred to as Brother (Bro) and Big Brother (Bgb), have been identified as Runt-interacting proteins (Golling et al., 1996; Fujioka et al., 1996). Both Bro and Bgb interact with Runt to enhance its DNA-binding affinity in vitro. The *Bro* and *Bgb* transcripts are expressed at uniformly high levels throughout the embryo during the syncitial blastoderm and cellular blastoderm stages. These are the stages during which Runt is required for sex determination and segmentation. Based on this overlapping expression and on what is known about the interaction of CBFα and CBFβ in mammals, it is reasonable to speculate that the Bro and Bgb proteins function with Runt during these stages.

Here we use a number of approaches to address the functional importance of interactions between Runt domain proteins and the Brother proteins during *Drosophila* development. First, we examine the effects of a point mutation in Runt, which specifically disrupts interaction with the Bro and Bgb proteins. This mutant is non-functional in a number of in vivo assays, supporting the hypothesis that interaction with the Bro proteins is critical for Runt function. Experiments that examine the activity of this mutant Runt protein on *Sxl* activation further suggest that interaction with Bro is required for more than stimulation of DNA-binding. We also generate a *Drosophila* version of the dominant negative CBFβ::MYH11 protein. The resulting fusion protein interferes with *runt* activity in the pathway of sex determination as well as with the activity of *lz* during eye development. Moreover, we find that ectopic expression of the wild-type Bro proteins during eye development rescues an intermediate *lz* phenotype. These results, in conjunction with the phenotypes generated by expression of the Bro::SMMHC and Runt proteins during eye development, indicate that Bro dosage is limiting in this developmental context. These results establish the in vivo importance of interactions between Runt domain proteins and Bro/Bgb proteins, provide new insights on Bro protein function, and lay a foundation for further studies in the *Drosophila* model system.

MATERIALS AND METHODS

Generation of plasmid constructs

pBS:runt[G163R] was generated by PCR following the instructions in the ExSite PCR-based site-directed mutagenesis kit (Stratagene) with the oligonucleotide primers, 5′-GTCCATCAAGTGCCGCA-ACGACG-3′ and 5′-ACCAGGGTGCCATCGGGCACGTC-3′ on a pB:ED(Bam-8, ΔKS) template (Kania et al., 1990). Plasmid DNA with the desired mutation was identified by sequencing and a *Sal*I/*Xho*I fragment containing this mutation with no other alterations was subcloned into pB:ED(Bam-8, ΔKS) digested with *Sal*I and *Xho*I. pGBT9(-1):runt[G163R] (Chien et al., 1991), pQE30:runt[G163R] (Qiagen) and pCS2+:runt[G163R] (Rupp et al., 1994) were generated by inserting the entire *Bam*HI fragment of pBS:runt[G163R] into vectors digested with *Bam*HI. For pUAS-runt[G163R], the *Bam*HI fragment of pBS:runt[G163R] was inserted into pUAST (Brand and Perrimon, 1993) digested with *Bgl*II.

To create pGBT9:BroSMMHC, a PCR product was generated using oligonucleotide primers 5′-ATCCGAATTCAATACAGTTATAACG-TTAGACAT-3′ and 5′-ATCCGGATCCGGGTGGAGTCTGGGGC-GT-3′ on a pACT:Bro template (Golling et al., 1996). The resulting PCR product was digested with *Eco*RI and *Bam*HI and cloned into pGBT9. The resulting plasmid, referred to as pGBT9:Bro$_{PP}$, contains amino acids 1-159 of Bro. A second PCR product was generated with primers specific to the break point and the C terminus of human CBFβ-MYH11 fusion protein, respectively, on a pGEM:KL2 template (generous gift from Paul Liu, NHGRI, Bethesda). The resulting PCR product, which encodes amino acids 1527-1972 of the *MYH11* gene product, was digested with *Bam*HI and *Sal*I and ligated into pGBT9:Bro$_{PP}$ cut with *Bam*HI and *Sal*I to produce pGBT9:Bro::SMMHC. pCS2+:Bro::SMMHC and pUAS-Bro::SMMHC were generated with the *Eco*RI/*Sal*I fragment of pGBT9:Bro::SMMHC ligated to pCS2+ and to pUAST, each digested with *Eco*RI and *Xho*I.

Two hybrid assay and DNA-binding experiments

The two-hybrid assay was performed as described in Golling et al. (1996). The Runt and Runt[G163R] coding regions were subcloned into the vector pGBT9(-1). Ubiquitin and Bgb were cloned into pACT (Chien et al., 1991). pGAD10:Bro was described in Golling et al. (1996). After transformation and plating on selective medium without leucine and tryptophan, colonies that grew well were restreaked onto fresh selective medium without leucine, tryptophan and histidine, and containing 33 mM 3-aminotriazole, and then incubated for 5 days at 30°C.

The Runt, Runt[G163R] and Bro proteins with hexa-His tags were expressed in bacteria using the pQE30 (Qiagen) expression system. The procedures used for protein purification and electrophoretic mobility-shift assays were based on work described by Pepling and Gergen (1995) with a few modifications. First, the reaction mixture

without the DNA probe was preincubated for 2 hours on ice. After the addition of DNA probe, the reaction mixture was incubated for another 2 hours on ice. The DNA-protein complexes were electrophoresed on a 10% polyacrylamide gel containing 0.05% Nonidet P-40 in 0.25× TBE running buffer containing 0.05% Nonidet P40 at 60 V for 17 hours at 4°C. The DNA probe used was a 37-bp fragment of the polyomavirus enhancer A element containing a PEBP2 binding site (Kamachi et al., 1990).

Fly strains

NGT40 is the transformant strain which contains the Gal4 coding region fused to the *nanos* promoter, followed by the 3′ non-translated region of *tubulin* gene (D. Tracey and J. P. Gergen, unpublished). This Gal4 driver provides embryos with evenly distributed maternal *gal4* mRNA. *Sev-Gal4* strain, which expresses Gal4 under control of the *sevenless* promoter, was provided by Utpal Banerjee (UCLA) (FlyBase, 1998*). UAS-Bro*, *UAS-Bgb*, *UAS-Bro::SMMHC*, *UAS-runt[G163R]* and *hs-runt[G163R]* lines were generated using standard procedures for germ line transformation. In each case, multiple lines were examined for activity. hs-runt[G163R] was first constructed in the CaSper P-element transformation vector with an FRT polyadenylation cassette inserted between the hsp70 promoter and the translation start site of Runt[G163R] (Buenzow and Holmgren, 1995). The recovered female transgenic flies were mated to male flies carrying *Flp* recombinase transgene (Golic and Lindquist, 1989). The loss of the polyadenylation cassette was confirmed by PCR.

Quantitation of RNA expression levels

2-4 hour embryos were collected from crosses of homozygous *NGT40* females mated to homozygous *UAS-runt* or *UAS-runt[G163R]* males, respectively. Total RNA was isolated using a modification of a procedure for preparing RNA from adult flies (Ashburner, 1989). Preparation of RNA probes and the RNase protection assay followed established protocols (Dixon and McKinnon, 1994). An anti-sense radiolabeled RNA probe corresponding to the 5′ region of the *runt* mRNA that includes 71 nucleotides which are not contained in the *UAS-runt* transgenes was synthesized from a pBS:E25-5′(ΔNotI) template. This DNA construct is derived from pBS:E25 (Kania et al., 1990) with a *Not*I fragment removed. 7.5 μg of total RNA was used in each hybridization reaction. After RNase treatment, the samples were run on a denaturing 6% polyacrylamide gel. For quantitation, the autoradiograph of the gel was analyzed using the ImageQuant program (Molecular Dynamics).

Sxl activation assay

The RNA injection procedure used to investigate *Sxl* activation was described in Kramer et al (1998). RNAs were synthesized in vitro from the appropriate pCS2+ DNA templates. The concentration of mRNA used for injection was 100-200 ng/μl. Injection of mRNA was directed to the middle region of embryos homozygous for the Sxl_{Pe}-*lacZ* reporter gene. After injection, embryos were aged until the late cellularization stage. After fixation and dehydration, embryos were stored in methanol before subjection to standard in situ hybridization, as described in Klingler and Gergen (1993). In these experiments, female embryos were identified on the basis of strong Sxl_{Pe}-*lacZ* expression at the poles, where *runt* is not required for female-specific expression.

Analysis of eye phenotypes

For examining the effects of Brother, Big Brother and Bro::SMMHC on the lz^{ts} eye phenotype, lz^{ts114}/*Binsinscy*; *Sev-Gal4*/*Cyo* females were crossed to homozygous *UAS-Bro*, *UAS-Bgb* and *UAS-Bro::SMMHC* males, respectively. (lz^{ts} flies were gifts from U. Banerjee, UCLA; Gupta and Rodrigues, 1995.) Progeny were collected at room temperature for 5-day intervals and then shifted to 25°C. Adult males were collected and examined for their eye phenotype. To examine the effect of Bro::SMMHC on eye patterning, males homozygous for *UAS-Bro::SMMHC* transgenes were mated to females heterozygous for *Sev-Gal4*. The cross was carried out at 25°C. Adult flies carrying both transgenes were characterized for their eye phenotype. To examine the consequence of ectopic expression of Runt with or without coexpression of Brother and Bro::SMMHC, females homozygous for *UAS-runt*, *UAS-runt*; *UAS-Bro* and *UAS-runt*; *UAS-Bro::SMMHC* were mated to males heterozygous for *Sev-Gal4*. The crosses were carried out at 25°C.

RESULTS

A Runt mutant that does not interact with Bro proteins

In order to investigate the functional importance of partner protein interaction for Runt, we generated a mutant derivative of Runt that is specifically impaired for interaction with the Bro proteins. Random mutagenesis of the Runt domain of CBFA1 identified mutations that affect the interactions with DNA and/or with the CBFβ partner protein (Akamatsu et al., 1997). Among these, one mutation is particularly interesting because it blocks interaction with CBFβ without affecting DNA binding activity. This mutation, G151R, is a replacement with an arginine by a glycine that is conserved in all Runt domain proteins. The corresponding point mutation in Runt, G163R, was generated with PCR-based site directed mutagenesis and then examined for its effects on Runt's in vitro activities.

The yeast two-hybrid assay was used to investigate the effects of the G163R mutation on the interaction between Runt and the Bro proteins. In this assay, interaction between the Runt and Bro proteins results in activation of a *His3* reporter gene, which allows for growth on plates lacking histidine (Fig. 1A). In contrast, no interaction between Runt[G163R] and Bro or Bgb was detected in the same assay. Interaction of Runt[G163R] with Ubiquitin, another Runt interacting protein (Golling et al., 1996), was detected, indicating the Runt[G163R] protein is expressed in these yeast transformants. To examine whether Runt[G163R] retains DNA binding activity, an electrophoretic mobility shift assay was performed in the presence or absence of the Bro protein. Purified proteins were incubated with a DNA probe containing a consensus binding site for Runt domain proteins. In the absence of Bro, Runt (WT) and Runt[G163R] showed weak but comparable DNA binding activity (Fig. 1B, lanes 2 and 4). However, in the presence of Bro, only Runt but not Runt[G163R] showed increased DNA binding activity and formation of a more slowly migrating DNA-protein complex (Fig. 1B, lanes 3 and 5). These data indicate that Runt[G163R] has a similar intrinsic DNA binding activity to Runt. Moreover, the data provide further evidence that Runt[G163R] does not interact with Bro. From the results of these in vitro assays, we conclude that the G163R mutation specifically disrupts the interaction between Runt and the Bro proteins without affecting the overall conformation of the Runt protein. This makes Runt[G163R] a valuable tool for investigating the importance of Bro protein interaction for the in vivo regulatory function of Runt.

The Runt[G163R] protein is defective in regulating gene expression in vivo

We used an ectopic expression assay to compare the activity of the Runt and Runt[G163R] proteins. Expression was driven

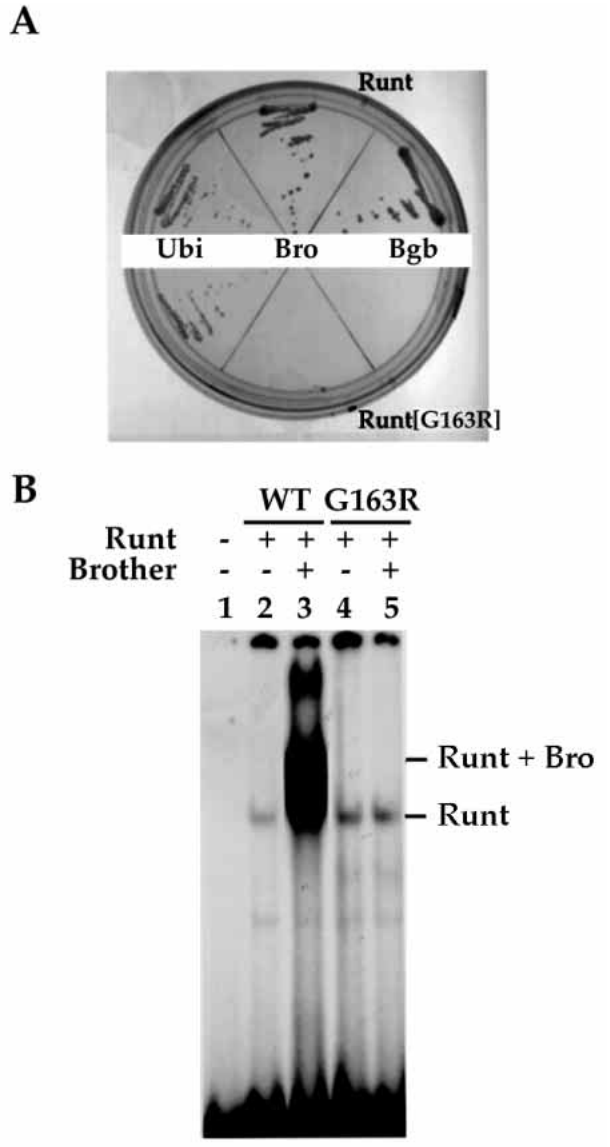

Fig. 1. A single point mutation of Runt, G163R, affects dimerization with Bro/Bgb but not DNA binding activity. (A) A two-hybrid assay was used to detect the interaction of Runt or Runt[G163R] and Bro or Bgb proteins. Interaction between two proteins was visualized by growth of yeast transformants on plates lacking histidine. Growth was detected for Runt but not Runt[G163R] when they were cotransformed with Bro or Bgb. Both Runt and Runt[G163R] showed interaction with Ubiquitin (Ubi), a protein that interacts with Runt in a Runt domain-independent manner. (B) Bacterially expressed proteins were incubated with DNA probe containing a binding site for Runt domain proteins. Then the DNA binding activity was tested in an electrophoretic mobility-shift assay. Runt and Runt[G163R] bind to DNA at a comparable efficiency in the absence of Bro (lanes 2 and 4). In the presence of Bro, Runt shows increased DNA binding affinity and an upper DNA-protein complex is detected. However, these phenomena are not observed when Runt[G163R] is used in this assay (lanes 3 and 5). This autography is overexposed to visualize the weak shifted bands. The much slower migrating band in lane 3 may be a multimer of proteins.

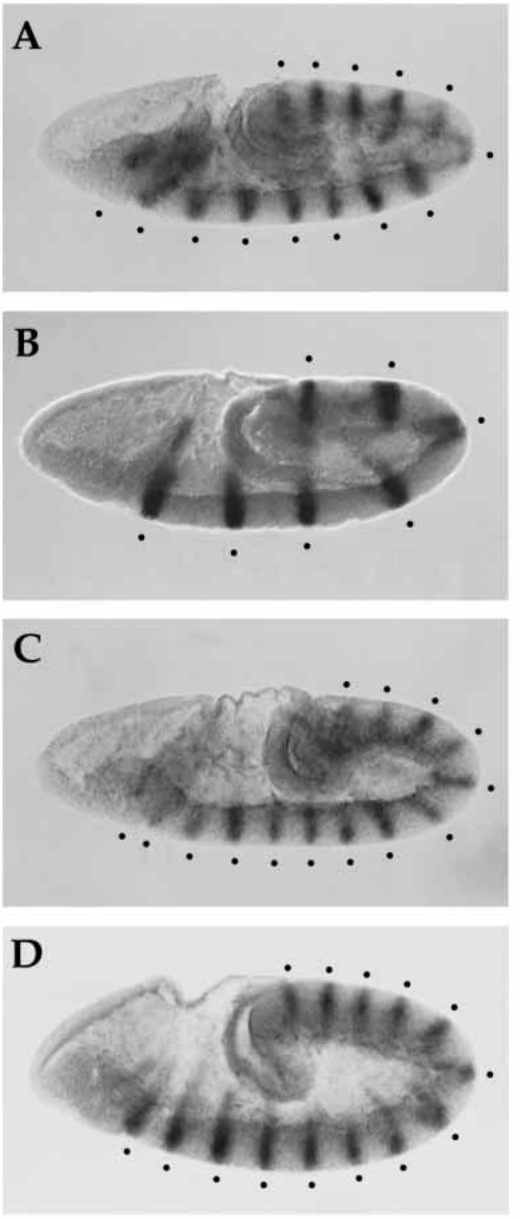

Fig. 2. Runt[G163R] does not have the ability to repress the odd-numbered *en* stripes. The expression of *en* in embryos ectopically expressing Runt or RuntG163R was visualized by in situ hybridization with an *en* ribo-probe. In this and the following figures, embryos are oriented with the anterior left and dorsal up. (A-C) Embryos were collected from females carrying the *NGT* driver. The embryo in (A) is from a cross with a male that does not contain an *UAS-runt* gene, and shows the normal 14-stripe *en* expression pattern. (B) Ectopic expression of Runt in the embryos heterozygous for the *UAS-runt*^{232} chromosome leads to repression of the odd-numbered *en* stripes. (C) Ectopic expression of Runt[G163R]$^{2\text{-}1}$ does not affect *en* expression. (D) The expression pattern of *en* is also not altered in embryos that express Runt[G163R] from a heat-inducible transgene. Expression of *hs-runt[G163R]* transgene is higher than that of *NGT* driven genes (data not shown).

using fly lines that maternally express GAL4, which allows for uniform expression during the cellular blastoderm stage (D. Tracey and J. P. Gergen, unpublished). Ectopic expression of Runt resulted in partial to complete lethality, depending on the *UAS-runt* lines used for the experiments. In contrast, ectopic expression of Runt[G163R] had no effect on viability (Table

Table 1. Lethal effects of ectopic Runt and Runt[G163R] expression

UAS-runt transgene	Adult viability[a] Female	Adult viability[a] Male	Embryonic viability[b]	RNA expression level[c]
runt				
U15	0	0	0	2.75±0.22
22	0	0	0	1.49±0.04
232	0	0	0	1.00±0
U14	77	26	58	0.52±0.01
runt[G163R]				
2-1	100	100	98	1.13±0.07
3-2	100	100	98	1.02±0.05

[a]Result is presented as percentage of flies carrying *UAS-runt* transgene/flies without *UAS-runt* transgene. Progeny were scored from crosses between males heterozygous for either *UAS-runt* or *UAS-runt[G163R]* and the appropriate balancer chromosome with females homozygous for the maternal Gal4 driver, *NGT40*. A minimum of 150 flies were scored for each cross.

[b]Result is expressed as percentage of embryos hatched. Males homozygous for either *UAS-runt* or *UAS-runt[G163R]* were crossed to females homozygous for the *NGT* driver. Results were pooled from two or three separate experiments. A minimum of 210 embryos were scored for each cross.

[c]Embryos collected from the crosses described in [b] were used in an RNase protection assay. The RNA expression level of each transgenic fly line was determined relative to the endogenous *runt* gene and these ratios were normalized using the *UAS-runt*^{232} as a standard. Values are means of three independent experiments ± s.d.

1). An RNase protection assay was used to demonstrate that these results are not due to differences in the RNA expression levels of the different transgenes (Table 1). The RNA expression levels of the two *runt[G163R]* lines used in these experiments were comparable to that of *UAS-runt*^{232}, a line that is fully lethal. We also examined the relative stability of the different proteins by western blotting and immunohistochemistry (data not shown) and found no difference between Runt and Runt[G163R].

The lethality associated with ectopic expression in these experiments is a crude assay for Runt function. In order to further characterize the activity of the Runt[G163R] protein, we examined the response of several Runt target genes to ectopic expression (Tsai and Gergen, 1994; Aronson et al., 1997). The most sensitive targets of Runt are the odd-numbered stripes of *engrailed* (*en*) expression (Fig. 2B). Ectopic expression of Runt[G163R] had no discernible effect on *en* expression (Fig. 2C), even at levels that are fivefold greater than required for repression of *en* by the wild-type Runt protein. To determine whether Runt[G163R] retains any residual activity we used heat-shock driven ectopic expression assay. The high levels of Runt expression obtained by this method cause alterations in the expression of other pair-rule genes in addition to *en* (Tsai and Gergen, 1994). Even under these conditions, the pattern of *en* expression as well as that of *even-skipped* and *fushi tarazu* in embryos expressing Runt[G163R] was indistinguishable from that of wild-type embryos (Fig. 2A,D; data not shown). These results indicate that Runt[G163R] is incapable of regulating expression of several of Runt's targets in the pathway of segmentation.

Runt[G163R] has a dominant negative effect on *Sxl$_{Pe}$* activation

In addition to functioning during segmentation, Runt also regulates the expression of the *Sxl* gene in the pathway of sex determination. Experiments using an in vivo mRNA injection assay demonstrate that DNA binding by Runt is essential for *Sxl$_{Pe}$* activation (Kramer et al., 1998). The same assay was used to investigate the effect of the Runt[G163R] mutation on *Sxl$_{Pe}$* activation. In vitro synthesized mRNAs encoding the Runt and Runt[G163R] proteins were injected into the central region of embryos carrying a *Sxl$_{Pe}$-lacZ* reporter gene. As has been described previously, injection of *runt* mRNA resulted in ectopic *lacZ* expression in male embryos without affecting expression in female embryos (Fig. 3A,C; Table 2). In contrast, when *runt[G163R]* mRNA was injected, no ectopic *Sxl$_{Pe}$-lacZ* expression was detected in male embryos (Fig. 3B; Table 2). This indicates that the Runt[G163R] is incapable of activating *Sxl$_{Pe}$*, suggesting that the interaction between Runt and Bro/Bgb is required for *Sxl$_{Pe}$* activation. Interestingly, we observed dominant interference of *Sxl$_{Pe}$* activation in female embryos injected with *runt[G163R]* mRNA (Fig. 3D; Table 2). The implications of this result will be discussed further below. Taken together, these several in vivo assays demonstrate that the Runt[G163R] protein is dysfunctional and strongly suggest that the interaction with Bro/Bgb is essential for Runt's function in embryogenesis.

A dominant negative form of Bro interferes with the activation of *Sxl$_{Pe}$*

To further address the biological significance of the interaction between Runt and Bro/Bgb, we expressed the CBFβ::MYH11

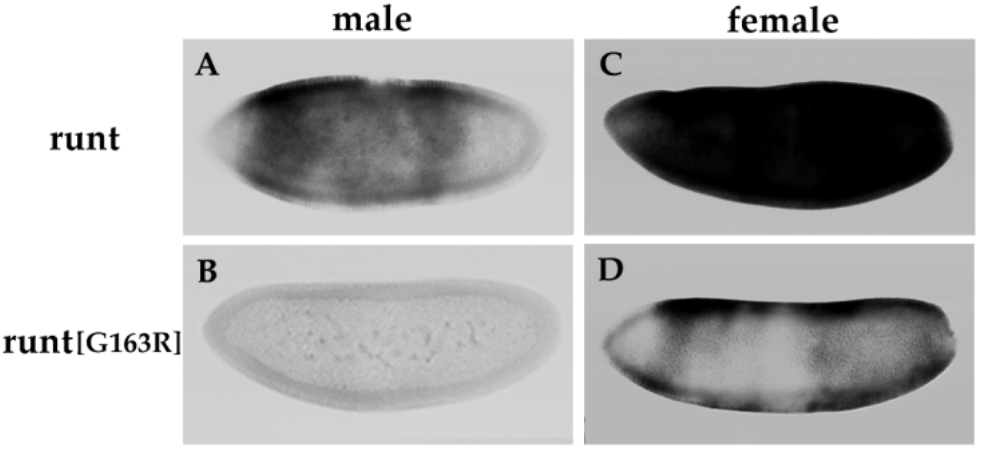

Fig. 3. Runt[G163R] is unable to activate *Sxl$_{Pe}$-lacZ* expression. Syncytial blastoderm stage embryos were injected with mRNA and expression of a *Sxl$_{Pe}$-lacZ* reporter gene was detected by in situ hybridization. (A,C) Injection of WT *runt* activates the expression of *Sxl$_{Pe}$-lacZ* in a broad central domain in male embryos without affecting the expression pattern in female embryos. (B,D) Injection of *runt[G163R]* does not show detectable activation of *Sxl$_{Pe}$-lacZ* in male embryos, but disrupts *Sxl$_{Pe}$-lacZ* expression in female embryos. The embryos shown in this figure are representative of typical strong phenotypes.

Table 2. Runt[G163R] and Bro::SMMHC negatively interfere with Sxl_{Pe}-*lacZ* expression

Total number of embryos scored	Injected mRNA	% embryos with *lacZ* pattern: Uniform activation[a]	Localized repression[b]	Localized activation[c]	No expression[d]
135	Water	47	0	0	53
387	*runt*	45	0	27	28
572	*runt[G163R]*	35	12	0	53
335	Bro::SMMHC	18	24	0	58
312	*runt+Bro::SMMHC*	36	11	18	35

[a]These embryos had dark uniform staining throughout the embryo except in pole cells.
[b]Localized repression was identified as weak to no staining in the central region but strong staining at the poles.
[c]These embryos showed weak to moderate staining in the central region but had no staining at the poles.
[d]These embryos showed no staining anywhere.

fusion protein in *Drosophila* using various assays. Expression of this fusion protein in the mouse produces embryonic lethal phenotypes similar to loss-of-function mutations of either *Cbfb* or *Cbfa2*. However, we did not detect any effect associated with ectopic expression of this mammalian fusion protein in *Drosophila*. The in vitro complex of Runt with mammalian CBFβ is less stable than the corresponding complex of Runt with the *Drosophila* Bro protein (Golling et al., 1996). We reasoned that these differences could account for the inactivity of the CBFβ::MYH11 fusion protein in our assays. Therefore, we decided to generate a presumptive dominant negative Bro by replacing the CBFβ portion of the CBFβ::MYH11 fusion protein with the analogous portion of Bro. This construct, named Bro::SMMHC, consists of the first 159 amino acids of Bro fused to the coiled-coil domain of human smooth muscle myosin heavy chain gene. To test whether Bro::SMMHC has a dominant negative effect on Sxl_{Pe} activation, the mRNA injection assay was performed. Injection of *Bro::SMMHC* mRNA interferes with Sxl_{Pe} activation in female embryos (Fig. 4C; Table 2). As expected, this construct does not activate Sxl_{Pe} in male embryos (Fig. 4A). To test whether this dominant negative interference is due to sequestering of Runt, we coinjected *Bro::SMMHC* and *runt* mRNA. The interference of Sxl_{Pe} activation by Bro::SMMHC is reduced in female embryos that are coinjected with *runt* (Fig. 4D; Table 2). Coinjection of *Bro::SMMHC* mRNA also reduces the ectopic activation of Sxl_{Pe} in male embryos compared to those injected with *runt* alone (Fig. 4B; Table 2). These results indicate that the Bro::SMMHC fusion protein functions as a dominant negative and provide further evidence for interactions between the Runt and Bro proteins in vivo.

Brother protein interactions with the Runt domain gene *lozenge*

A second *Drosophila* Runt domain protein, Lz, participates in patterning the eye. We expressed Bro::SMMHC in the developing eye under the control of a *Sev-Gal4* driver to determine if this dominant negative protein interferes with *lz* function. Expression of Bro::SMMHC causes mild patterning defects in the eyes of flies that are wild-type for *lz* (Fig. 5A). Further, the phenotype produced by a temperature-sensitive *lz* allele at an intermediate temperature is much more severe in flies that express the Bro::SMMHC protein (Fig. 5B,C). These results suggest that *lz* activity may provide a sensitive assay for Bro protein function. Consistent with this, we find that expression of *UAS-Bro* and *UAS-Bgb* transgenes under the control of a *Sev-Gal4* driver rescues the phenotype of the lz^{ts} mutation at 25°C (Fig. 5D,E). Similar observations have also been made by Utpal Banerjee and colleagues in flies where *sev* control elements are used to drive *Bgb* expression directly (U. Banerjee, personal communication). We examined the activities of multiple *UAS-Bro* and *UAS-Bgb* lines and found that Bro consistently rescues the *lz* phenotype better than Bgb. This suggests that Bro is a more effective partner for Lz, although we cannot rule out that the difference is due to protein expression levels.

The above observations provide strong evidence for functional interactions between Bro/Bgb and Lz. Interestingly,

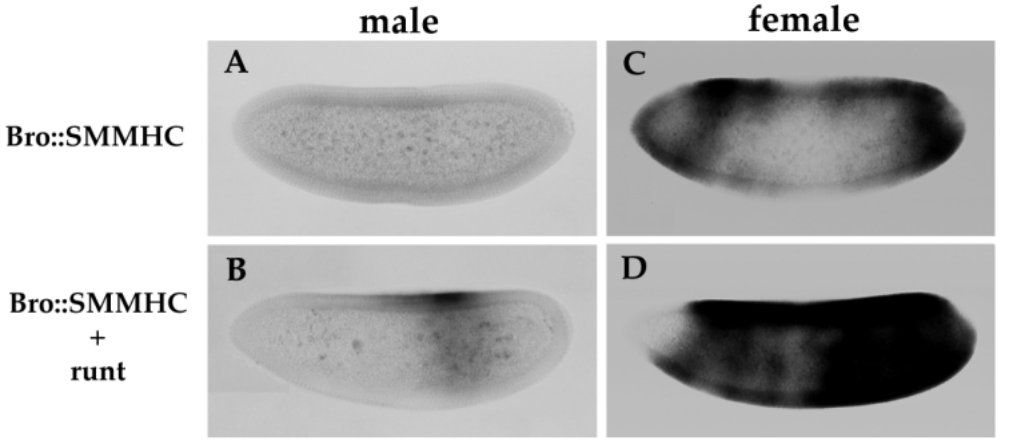

Fig. 4. Bro::SMMHC has dominant negative interference on Sxl_{Pe}-*lacZ* expression. Expression of the Sxl_{Pe}-*lacZ* reporter gene was visualized with in situ hybridization in embryos injected with RNA. The embryos shown here are representatives of typical strong phenotypes. (A,C) Injection of *Bro::SMMHC* does not activate Sxl_{Pe}-*lacZ* expression in males but does result in repression of Sxl_{Pe}-*lacZ* expression in females. (B) Coinjection of *Bro::SMMHC* mRNA with *runt* reduces the efficiency of Sxl_{Pe}-*lacZ* activation in male embryos compared to male embryos injected solely with *runt* mRNA (compare with A). (D) Similarly, coinjection of these two mRNAs into female embryos results in a more normal Sxl_{Pe}-*lacZ* expression pattern than observed in female embryos injected solely with *Bro::SMMHC* mRNA (compare with C).

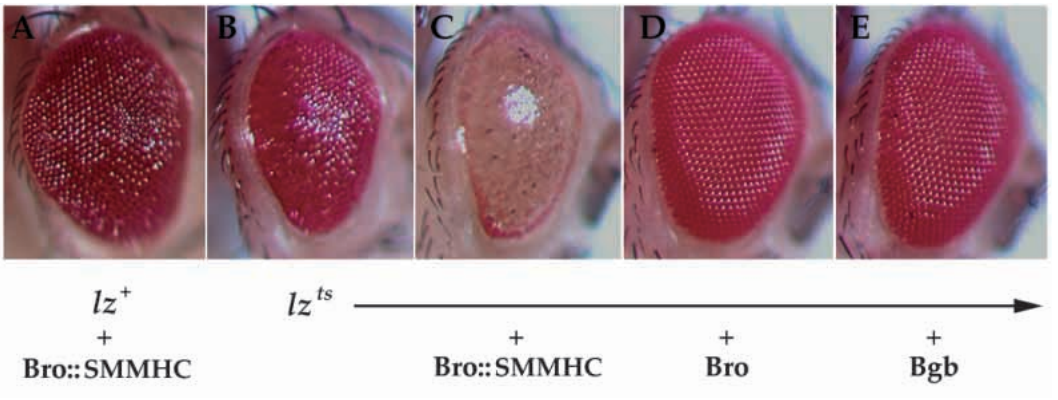

Fig. 5. Effect of Bro protein expression on eye patterning. Adult eyes were viewed with a stereo dissecting microscope. (A) An eye from a female is shown here. Ectopic expression of Bro::SMMHC with the *Sev-Gal4* driver in wild-type flies results in rough eyes. (B-E) Male progeny were collected from crosses in which males homozygous for UAS transgenes were mated to females heterozygous for lz^{ts114} and *Sev-Gal4*. (B) An eye of a lz^{ts114} hemizygous male shows disrupted facets and reduced pigment at 25°C. (C) This eye phenotype is enhanced when Bro::SMMHC is ectopically expressed. (D) The *lz* eye phenotype is completely rescued by ectopic expression of Bro. (E) A nearly normal ommatidial lattice is obtained when Bgb is expressed in the same manner.

and in contrast to the results with *lz*, the intermediate phenotypes produced by several *runt* hypomorphic mutants, including a temperature-sensitive allele, are not modified by ectopic expression of *UAS-Bro* or *UAS-Bgb* transgenes (data not shown). Furthermore, expression of *UAS-Bro::SMMHC* has no obvious effect on *runt* activity during embryogenesis (data not shown). The mRNA injection experiments described above indicate that the Bro::SMMHC fusion protein interacts with Runt in the pathway of sex determination. The explanation for these different results seems likely to be due to the levels of ectopic expression, which are greater in the RNA injection experiments than through use of the Gal4/UAS expression system. The *Bro* and *Bgb* mRNAs are expressed at relatively high levels during the blastoderm stage (Golling et al., 1996) and the level of ectopic Bro::SMMHC expression would have to approach this level in order to produce a clear phenotype. In the context of this explanation, the sensitivity of the *lz* phenotype to both the dominant negative activity of Bro::SMMHC and the suppressing activity of the wild-type Bro and Bgb proteins suggests that partner protein activity is lower and possibly limiting during eye development.

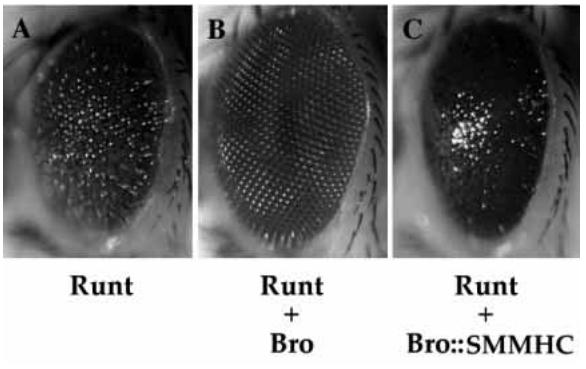

Fig. 6. Runt interferes with Lz by competing for Bro proteins. The eyes of flies carrying *Sev-Gal4* and various transgene(s) were viewed with stereo dissecting microscope. (A) Ectopic expression of Runt with the *Sev-Gal4* driver results in an *lz*-like eye phenotype. (B) This eye phenotype is suppressed when Brother was coexpressed with Runt. (C) A severe eye phenotype is produced when Bro::SMMHC and Runt were coexpressed.

The hypothesis that Bro protein levels are limiting during eye development is further supported by experiments that involve expression of Runt under the control of the *Sev-GAL4* driver. Expression of Runt in wild-type eye discs results in a phenotype similar to that of *lz* mutants (Fig. 6A). In contrast, expression of Runt[G163R] does not result in any detectable abnormalities in the eye (data not shown). One explanation for these results is that Runt interferes with Lz by competing for the Bro and/or Bgb proteins. To test this possibility, Bro was coexpressed with Runt in wild-type eye discs. The resulting eyes are normal (Fig. 6B). This result is consistent with the competition model. In contrast, a more extreme eye phenotype would be expected in most other models for interference by Runt. Finally, coexpression of Bro::SMMHC and Runt resulted in an eye phenotype that is even more severe than that produced by expression of either protein alone (Fig. 6C). Our interpretation of this result is that Bro::SMMHC sequesters both Runt and Lz, and that the Runt protein that remains further reduces Lz function by competing for the limiting Bro/Bgb proteins.

DISCUSSION

Importance of Bro proteins for Runt domain proteins' function in vivo

In this paper, we provide several lines of evidence to demonstrate the biological significance of the interaction between *Drosophila* Runt domain proteins and the Bro and Bgb proteins. First, a Runt mutant, Runt[G163R], which does not interact with Bro/Bgb but retains in vitro DNA binding activity, is dysfunctional in several different in vivo assays. Second, Bro::SMMHC, a *Drosophila* version of CBFβ::MYH11, has a dominant negative interfering effect on Runt-dependent Sxl_{Pe} activation. These observations strongly suggest that interactions with the Bro/Bgb proteins are required for Runt's function during embryogenesis. In addition, we find that expression of Bro::SMMHC interferes with the function of the Runt domain gene *lz* in the developing eye, whereas the patterning defects associated with reduced *lz* function are suppressed by expression of either the Bro or Bgb proteins. These results strongly suggest that interactions with Bro and/or Bgb are important for Lz function in eye patterning.

Bro/Bgb dosage is limiting in eye discs but not in embryos

Several observations suggest that the relative dosage of the Bro/Bgb proteins in eye discs and in embryos is different. First, The effects caused by expression of the Bro::SMMHC fusion protein in flies that are wild-type for *runt* and *lz* provides a clear indication of differences between the embryo and the eye. Expression of Bro::SMMHC with the *Sev-Gal4* driver gives rise to patterning defects in the eye. In contrast, the expression of Bro::SMMHC that is driven by maternally expressed Gal4 produces no phenotype in embryos. The absolute levels of ectopic expression produced by these two Gal4 drivers in these two different developmental contexts are not known. However, based on the results of RNase protection assays, the expression levels achieved with the maternal Gal4 system are greater than the levels of endogenous *runt* expression. The sole assay in which we detect a dominant negative effect of Bro::SMMHC in the embryo is in the Runt-dependent activation of Sxl_{Pe} transcription. This effect is produced by injection of mRNA, which allows ectopic expression levels greater than can be obtained using the Gal4 system. Our interpretation of these results is that high levels of Bro::SMMHC are required to compete with the abundant Bro and Bgb proteins in the embryo (Golling et al., 1996).

A second indication that Bro/Bgb dosage is not limiting in the embryo comes from the observation that the intermediate phenotypes produced by several *runt* hypomorphic mutations are not modified by ectopic expression of *UAS-Bro*, *UAS-Bgb* or *UAS-Bro::SMMHC*. These intermediate phenotypes are detectably altered by other relatively subtle perturbations, such as disruption of dosage compensation in female embryos (Gergen, 1987). An alternative explanation is that the Bro and Bgb proteins expressed from these transgenes are not capable of functional interactions with Runt. It will be necessary to obtain mutations that specifically affect Bro and Bgb activity in order to distinguish between these two explanations

A final line of evidence that Bro/Bgb dosage is limiting in the eye comes from experiments that involved ectopic expression of Runt and Lz. Ectopic expression of Runt in eye discs results in a phenotype that resembles that of *lz* mutants. This phenotype is suppressed by coexpression of Bro, suggesting that Runt interferes with Lz by competing for limiting quantities of Bro proteins. In contrast, expression of Lz in embryos, although lethal, does not interfere with Runt's function in segmentation (J. P. Gergern, unpublished data). These observations strongly support the idea that the dosage of Bro/Bgb proteins is limiting in eye discs but not in embryos. The sensitivity of the *lz* phenotype to Bro and Bgb dosage further suggests that mutations in these genes can be isolated in screens for modifiers of the *lz* eye phenotype.

The requirement of Bro for Runt function is not restricted to enhancing DNA binding

We observe repression of Sxl_{Pe} expression in female embryos injected with *runt[G163R]* mRNA. This dominant negative activity indicates that the Runt[G163R] protein interacts with some other factor(s) in the *Drosophila* embryos in a manner that interferes with the activity of the wild-type Runt protein. In contrast to this, no dominant negative interference is observed when *runt[CK]*, a Runt derivative that is specifically impaired for DNA-binding, is used in this assay (Kramer et al., 1998). If the Runt[G163R] protein was interfering by competing for interaction with some other limiting protein factors then Runt[CK] protein would also be expected to behave as a dominant negative. Taken together, these results suggest that DNA binding is required for the dominant negative activity of Runt[G163R]. This is somewhat surprising as the prevailing view, primarily from in vitro experiments, has been that the central function of the Bro/Bgb and CBFβ proteins is to enhance DNA-binding by the Runt domain proteins. Our data strongly suggest that the Bro proteins have other functions in addition to enhancement of DNA binding by Runt. What then might be the other functions of the Bro/Bgb proteins? One possibility is that Bro induces a conformational change in Runt that is required for transcriptional activation. Runt/Bro complexes induce a bend in DNA that is greater than that observed by binding of Runt alone (Golling et al., 1996). Perhaps DNA-bending is critical for interactions between Runt and other transcription factors on the Sxl_{Pe} promoter. An alternative possibility is that Bro/Bgb may be a bridge between Runt and other proteins that are critical for transcription regulation. In this model Runt[G163R] would compete for binding to the early promoter region of Sxl_{Pe}-*lacZ* but when bound would fail to activate transcription because other Bro-interacting proteins are not recruited. In a two-hybrid screen for Bro-interacting proteins we have identified a number of proteins that appear to be members of the trithorax group of transcriptional regulators (G. Golling, personal communication). Trithorax group proteins have been implicated as having widespread roles in transcription activation in *Drosophila* development and it is attractive to speculate that recruitment of such proteins by Runt and Bro contributes to the activation of *Sxl* transcription. It is clear from the results presented here that interactions between Runt domain proteins and Bro/Bgb/CBFβ proteins are important for the functions of these conserved transcriptional regulators. Experiments that further address the functions of the Bro/Bgb and CBFβ proteins will be essential for understanding the mechanisms that account for the pivotal regulatory roles of these proteins in diverse developmental contexts.

We are particularly grateful to Utpal Banerjee for the contribution of lz^{ts} *Drosophila* strains and sharing results of eye phenotype rescue experiments prior to publication. We thank Paul Liu for providing *Cbfb::SMMHC* cDNA and thoughtful suggestions. Information provided by Katsuya Shigesada on the generation of a Brother-interacting-defective form of Runt is greatly appreciated. Sunita G. Kramer provided invaluable technique support for processing mRNA injection experiments. Xiangqun Ning's assistance with some of the mRNA injection experiments and Claudia Brunner's assistance with some of the transgenic fly generation is also appreciated. We thank Dan Tracey, John Wheeler, Mary Wines and Kathy Wojtas for comments on the manuscript. This work was supported by NIH grants to J.P.G.; J.P.G. was a recipient of a Faculty Research Award from the American Cancer Society (FRA428).

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